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PRODUCTION AND SPECIFICITY OF ANTIBODIES DIRECTED TOWARD 3,4,5-TRIMETHOXYPHENYLETHYLAMINE, 3,4-DIMETHOXYPHENYLETHYLAMINE AND 2,5-DIMETHOXY-4-METHYLAMPHETAMINE*

HELEN VAN VUNAKIS, HILDA BRADVICA, PHILIPPE BENDA and LAWRENCE LEVINE

Graduate Department of Biochemistry, Brandeis University, Waltham, Mass., U.S.A.

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Abstract—Mescaline (3,4,5-trimethoxyphenylethylamine) and 3,4-dimethoxyphenylethylamine were coupled to polyglutamic acid with the use of water-soluble carbodiimides. Complexes of these conjugates with methylated bovine serum albumin elicited in rabbits antibodies directed toward mescaline and 3,4-dimethoxyphenylethylamine. DOM (2,5-dimethoxy-4-methylamphetamine) was coupled to rabbit serum albumin with glutaraldehyde. This conjugate elicited in rabbits antibodies directed toward DOM. The specificities of the antisera were examined by C′ fixation with the conjugates and inhibition of C′ fixation by various phenylethylamine analogues. With the mescaline immune system, antibody combining sites were complementary to the 3,4,5-trimethoxyphenyl moieties. With the 3,4-dimethoxyphenylethylamine immune systems, the complementarity between the antigenic determinants and the antibody combining sites was best with phenylethylamine derivatives containing only the 3,4-dimethoxy groups. With the DOM immune system, the antibody combining site recognized the 4-methyl-2,5-dimethoxyphenyl groups.

Within the past 4 yr, carbodiimides have been used to synthesize various antigenic conjugates composed of relatively large carriers and low molecular weight substances. The low molecular weight substances have included bradykinin and angiotensin,[1, 2] nucleotides[3–5] and oligonucleotides,[4] and 5-hydroxyindole acetic acid, a derivative of serotonin,[6] and the macromolecules have included proteins[1–4, 6] and synthetic polypeptides.[4, 5] Also within the past 4 yr, electrostatic complexes of methylated bovine serum albumin (MBSA) and negatively charged molecules (deoxyribonucleic acid,[7, 8] polyribonucleotides,[9] polysaccharides,[7] polyglutamic acid[10]) as well as phosphorylated bovine serum albumin and positively charged macromolecules (polylysine and polyornithine[11]) have been used to elicit antibody production to the negatively or positively charged macromolecules.

Antibodies specific to serotonin were obtained[12] by immunizing rabbits with conjugates in which serotonin was coupled to carrier proteins via position 1 of the indole

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ring by use of the Mannich formaldehyde reaction. Recently, glutaraldehyde has been used successfully to couple small peptides to proteins and render them antigenic.[13]

This article describes the production of antibodies to mescaline [3,4,5-trimethoxyphenylethylamine (TMPE), 3,4-dimethoxyphenylethylamine (DMPE) and 2,5-dimethoxy-4-methylamphetamine (DOM)]. Antibodies to the first two compounds were elicited by immunizing rabbits with a conjugate in which TMPE and DMPE were covalently linked to polyglutamic acid by carbodiimide, then complexed to MBSA, while antibodies to DOM were obtained by immunization with a DOM–glutaraldehyde–rabbit serum albumin conjugate synthesized according to the Reichlin procedure.[13] The specificities of these antisera were determined by complement fixation with the conjugates and inhibition of specific complement fixation with analogues of phenylethylamine.

MATERIALS AND METHODS

Materials. MescalineHCl[3,4,5-trimethoxyphenylethylamine (TMPE)] was purchased from the Sigma Chemical Co. and 3,4-dimethoxyphenylethylamine (DMPE) was purchased from the Aldrich Chemical Co. DOM (2,5-dimethoxy-4-methylamphetamine) was given to us by Dr. S. H. Snyder of the Johns Hopkins University. The other derivatives used in this study were the generous gifts of Dr. W. E. Scott of Hoffman-La Roche Pharmaceutical Co. These included *N*-methylmescaline, *N*-dimethylmescaline and 3,5-dimethoxyphenylamine. Poly-L-glutamic acid (PG) as the sodium salt was obtained from Pilot Chemical Co. and possessed a degree of polymerization equivalent of 520.

Two different water-soluble carbodiimides, one aliphatic [1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (CDI)] HCl, from Ott Chemical Co. and the other aromatic [1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide, metho-*p*-toluenesulfonate) (CMC)] from Aldrich Chemical Co., were used in this study.

Coupling of DMPE and TMPE to poly-L-glutamic acid (DMPE–PG or TMPE–PG). DMPE or TMPE was coupled via the primary amino group to the γ-carboxyl groups of polyglutamic acid by using water-soluble carbodiimides. In a representative synthesis, 25 mg DMPE and 25 mg poly-L-glutamic acid were dissolved in 0·5 ml distilled water and the pH was adjusted to 7·6. In the clear viscous solution was dissolved 50 mg of the aliphatic or 65 mg of the aromatic carbodiimide and the tubes were left to stand at room temperature for 18–24 hr. At the end of this time, the reaction mixture was quantitatively transferred to a dialysis bag and dialyzed against 2 l. of 0·15 M NaCl, (three changes for 3 days). The samples were removed quantitatively and the bag was rinsed with small portions of saline. The amount of polymer present was calculated from the starting weight and the final volume of the solution. The amount of TMPE and DMPE coupled per residue of glutamic acid was determined from spectral measurements. Although polyglutamic acid does not absorb in the range of 240–300 mμ, the extent of substitution determined from spectral measurement might be erroneous if the aromatic carbodiimide (CMC) were covalently bound to the polymer or if the spectral properties of the covalently linked phenylethylamines varied from those of the free phenylethylamines. However, the side reaction of the carbodiimide to form an N-substituted urea on the polymer was negligible and the linkage to the polymer did not involve the aromatic ring which is responsible for the absorption in the ultraviolet region.

Synthesis of rabbit serum albumin (RSA)–glutaraldehyde–DOM. Twenty mg RSA was dissolved in 2 ml of 0·1 M phosphate buffer, pH 7·0, and 6 mg DOM was then dissolved in the solution. A 25% glutaraldehyde solution was diluted 1/115 with water, and 1 ml was added dropwise with mixing. The reaction mixture was kept at room temperature for 2 hr. In the course of this incubation the solution became yellow. One-tenth ml of a 1 M lysine solution at pH 7·5 was added to react with the excess glutaraldehyde and the incubation continued for 1 hr. The sample was then dialyzed in the cold against several changes of 0·15 M NaCl for 48 hr. The amount of DOM coupled to the RSA was calculated from difference spectra and was found to be 14 μmoles DOM per μmole RSA. (We would like to thank Dr. Morris Reichlin for informing us of this useful procedure for synthesis of antigenic conjugates prior to publication.)

Immunization of rabbits. To 1 mg DMPE–PG or TMPE–PG in a 1·0-ml vol. was added 10 μl of 10% MBSA. The resulting flocculent suspension was prepared in an emulsion with complete Freund's adjuvant and injected into the toe pads and leg muscles of albino New Zealand rabbits. Three weeks later, the animals received a single intramuscular injection and were bled 5 days later. In the work reported in this article, only sera from rabbits 866 (anti-PG–TMPE) and 822 (anti-PG–DMPE) were used. For production of antibodies to DOM, 2 mg of the RSA–glutaraldehyde–DOM in complete Freund's adjuvant was injected into toe pads and intramuscularly. Two injections were given 2 weeks apart. The rabbits were bled 1 week later.

Serologic analyses. Complement (C′) fixation and inhibition of C′ fixation were performed by the procedure described by Levine.[14]

RESULTS

A schematic representation of the antigenic conjugate, TMPE–PG, is shown in Fig. 1. Two possible products are shown. In the desired product, the amino group of DMPE is coupled to the carboxyl group of PG through an amide bond. The formation of a stable N-substituted urea formed by condensation of the carbodiimide with the macromolecule may also take place. Thus, it is possible to elicit antibodies to the backbone, substituted urea and the coupled DMPE. To minimize the complications of antibodies to the substituted urea, two different carbodiimides have been used. The conjugate used for immunization was synthesized with an aliphatic carbodiimide (CDI), while the antigenic conjugate to be used for serologic analyses was synthesized with the aromatic carbodiimide (CMC). This precautionary procedure was not necessary, however, for the antisera contained few, if any, antibodies directed to the substituted urea. The absorption spectra of a representative sample of DMPE–PG and TMPE–PG are shown in Fig. 2. Also shown are the absorption spectra of the DMPE and TMPE used in the synthesis. The yields of several different preparations are given in Table 1.

Figure 3 depicts the C′ fixation curves with PG and PG–DMPE with one antiserum dilution. If, however, the antiserum is at five times greater concentration (1/100), a reaction between PG and anti-PG is observed. With the TMPE–PG immune systems, antibodies directed toward PG and TMPE could not be distinguished by antiserum dilution. Both PG and TMPE–PG react with anti-TMPE–PG at a 1/300 dilution of the antiserum (Fig. 4(A)). The C′ fixing activity of the antibodies to PG could be effectively eliminated by absorption of the antiserum with PG or by assaying the TMPE–PG immune system in the presence of excess PG. In the latter procedure, the

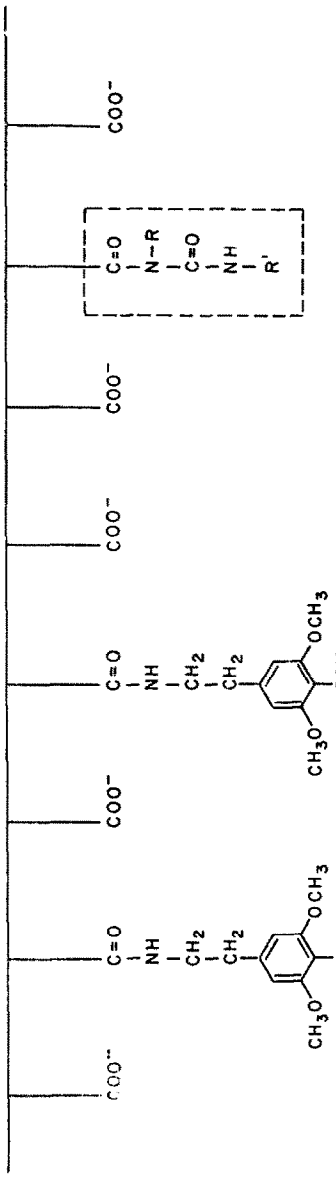


FIG. 1. Schematic representation of the product(s) formed when TMPE is conjugated to PG by Carbodiimide. See text for abbreviations.

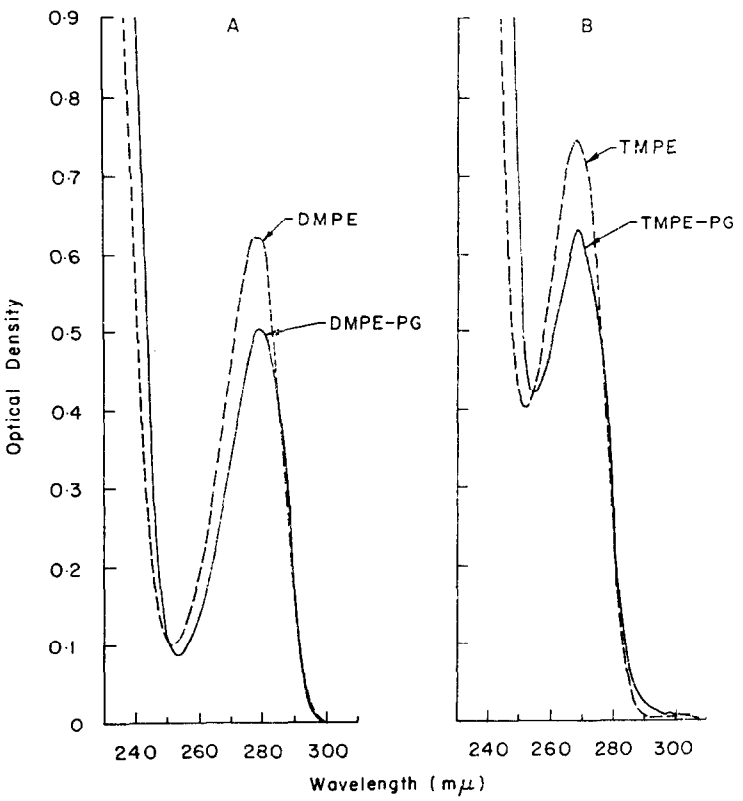


FIG. 2. Absorption spectra of (A) DMPE (- - -) and DMPE–PG (———) and (B) TMPE (- - -) and TMPE–PG (———).

TABLE 1. EXTENT OF COUPLING OF HAPTENS TO POLYGLUTAMIC ACID

Preparation	Moles of hapten/10 mole glutamic acid residues
1. Poly-L-Glu-CMC-TMPE*	2·7
2. Poly-L-Glu-CMC-DMPE	2·1
3. Poly-L-Glu-CDI-TMPE	4·7
4. Poly-D-Glu-CDI-TMPE	3·9
5. Poly-D-Glu-CDI-DMPE	3·6
6. Poly-L-Glu-CDI-DMPE	8·0
7. Poly-L-Glu-CDI-TMPE	3·7

* See text for abbreviations.

PG–anti-PG reaction is not observed due to the lack of C′ fixation in the antigen excess region of the C′ fixation curve. Thus, under these conditions, only the C′ fixation of antibodies directed toward TMPE could be measured with TMPE–PG. The activity of the TMPE–PG antiserum (at a 1/100 dilution) in the presence of excess PG is shown in Fig. 4(B).

The specificity of the DMPE–PG and TMPE–PG immune systems was tested by inhibition of the homologous systems by phenylethylamine analogues. With the

DMPE immune system, the reaction of 0·3 μg DMPE–PG with antiserum to DMPE–PG diluted 1/500 was inhibited and, with the TMPE–PG immune system, the reaction of 0·02 μg TMPE–PG and anti-TMPE–PG (diluted 1/100) in the presence of an excess of PG (10 μg) was inhibited. The inhibition data for the DMPE immune system are shown in Fig. 5. The homologous hapten, 3,4-DMPE, was the most effective inhibitor (1·0 μg inhibited 25 per cent). The presence of a methoxy substituent on the 5 position of the benzene ring reduced the inhibitory effectiveness about 100-fold, while the 2,5-methoxy or the 3-methoxy analogues were not inhibitory even at 500-μg levels.

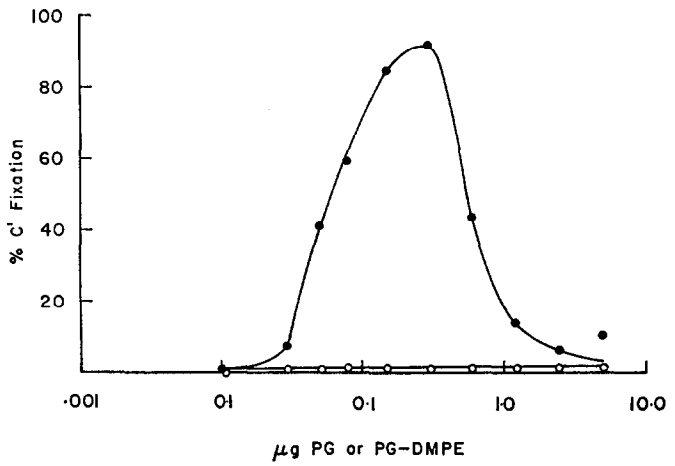


FIG. 3. C′ fixation reaction of PG–DMPE (●) and PG (○) with anti-PG–DMPE diluted 1/500.

The inhibition data for the TMPE immune system are shown in Fig. 6. All of the analogues having methoxy groups on the 3,4,5 positions of the benzene ring are of equal inhibitory effectiveness. Removal of one methoxy group from the 4 position or the 5 position reduces the inhibitory effectiveness at least 50-fold. The chemical structures of the analogues tested and their inhibitory activities for both immune systems are shown in Table 2.

The rabbits immunized with DOM–PG (made by the carbodiimide reaction) complexed to MBSA, failed to produce antibodies directed toward DOM. Two rabbits immunized with RSA–glutaraldehyde–DOM, however, did produce antibodies that showed specificity toward DOM, as shown in Fig. 7. Of four RSA conjugates tested, only RSA–glutaraldehyde–DOM reacted with anti-RSA–glutaraldehyde–DOM. RSA–glutaraldehyde–TMPE, RSA–glutaraldehyde–DMPE and RSA–glutaraldehyde–amphetamine were inactive. The inhibitory activities of DOM, DMPE, TMPE and amphetamine are shown in Fig. 7(b). Only DOM inhibited the RSA-glutaraldehyde–DOM immune system (50 per cent inhibition obtained with 50 μg DOM).*

The other analogues were tested at 100-μg levels and were not active. Thus, the antibodies appear to recognize the methoxy groups on the 2,5 positions and the methyl group on the 4 position of the benzene ring. More detailed studies of the specificity of the DOM immune system must await testing of the inhibitory activities of various amphetamine derivatives.

* After continued immunization, the RSA glutaraldehyde–DOM system is much more sensitive to inhibition by DOM; 50 per cent inhibition is obtained with 2 μg DOM.

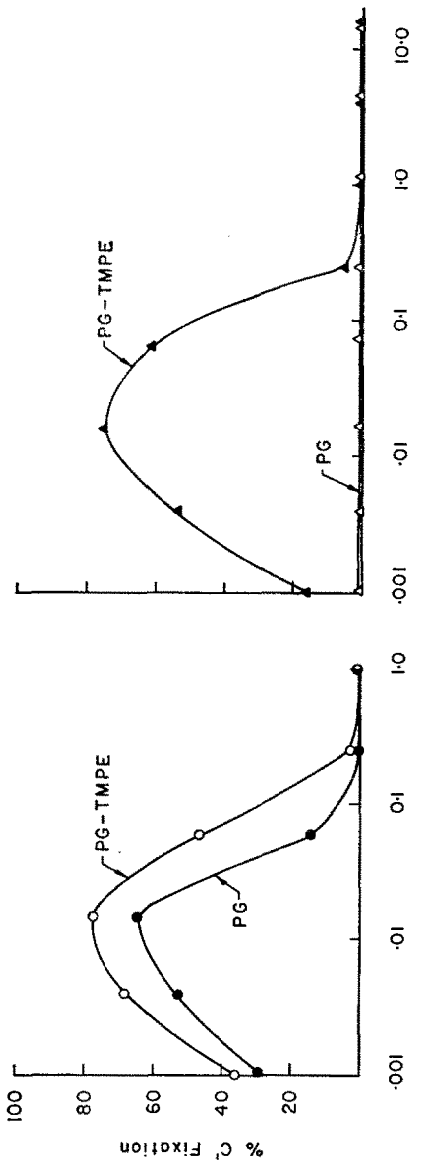
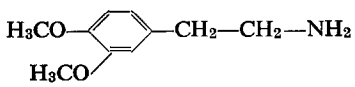
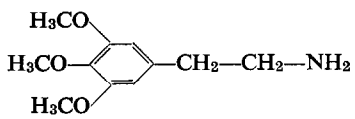
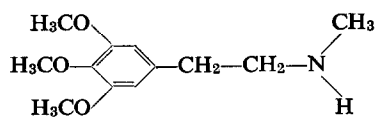
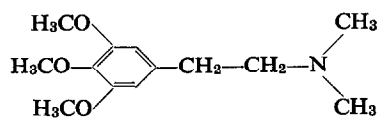
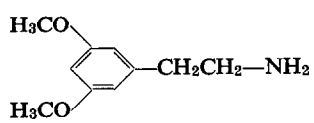
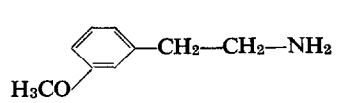
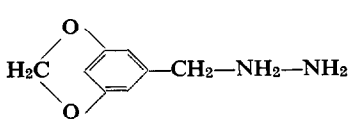
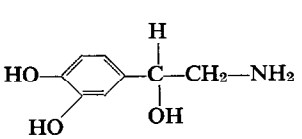
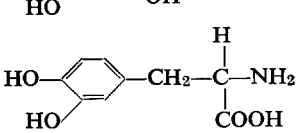


FIG. 4. C' fixation of (A) PG–TMPE (○) and PG (●) with anti-PG–TMPE diluted 1/300; (B) PG–TMPE (▲) and PG (△) with anti-PG–TMPE diluted 1/100 in the presence of 10 μg PG.

TABLE 2. INHIBITION OF DMPE–PG AND TMPE–PG IMMUNE SYSTEMS

Inhibitor		μg Required for 50% inhibition*	
Compound	Structure	DMPE immune system	TMPE immune system
3,4-Dimethoxyphenylethylamine (DMPE)		2·7	>50†
3,4,5-Trimethoxyphenylethylamine (Mescaline) (TMPE)		250	3·8
N-methylmescaline		300	5·0
N-dimethylmescaline		250	6·3
3,5-Dimethoxyphenylethylamine		>500‡	>50§
M-methoxyphenylethylamine		>500‡	>50§
Methylene dioxyphenylethylamine		>500‡	>50§
l-3,4-Dihydroxyphenylethanolamine (*l*-Norepinephrine)		>500‡	>50§
2-Amino-3(3,4-dihydroxylphenyl)-propanoic acid (Dopa)		>500‡	>50§

* Immune systems used.
† With 50 μg, 19 per cent inhibition.
‡ With 500 μg, no inhibition.
§ With 50 μg, no inhibition.

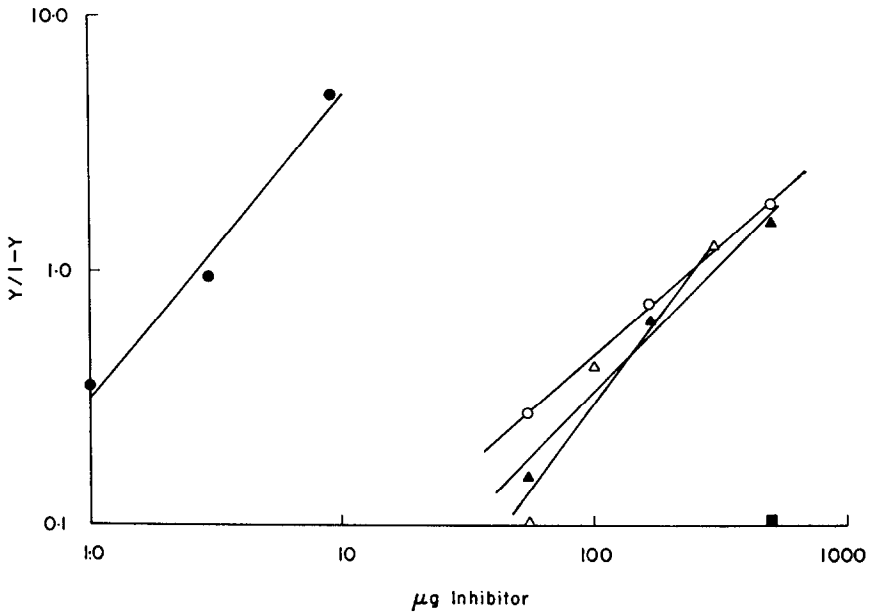


FIG. 5. C′ fixation inhibition of the reaction between 0·3 μg PG–DMPE and anti-PG–DMPE diluted 1/500 by DMPE (●), TMPE (△), *N*-dimethylmescaline (○), *N*-methylmescaline (▲) and 3,5-dimethoxyphenylethylamine (■). Y/1 − Y is plotted vs. micrograms of hapten (Y is the per cent inhibition of C′ fixation divided by 100).

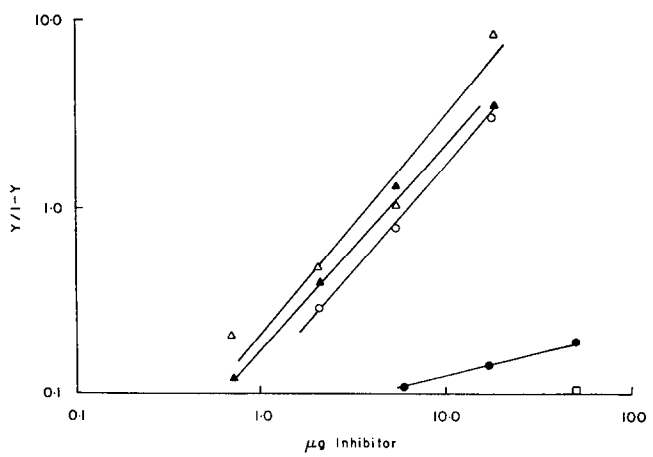


FIG. 6. C′ fixation inhibition of the reaction between 0·02 μg PG–TMPE and anti-TMPE diluted 1/100 in the presence of 10 μg PG by TMPE (△), *N*-methylmescaline (▲), *N*-dimethylmescaline (○), DMPE (●) and 3,5-dimethyoxyphenylethylamine (□). Y/1 − Y is plotted vs. micrograms of hapten. (Y is the per cent inhibition of C′ fixation divided by 100).

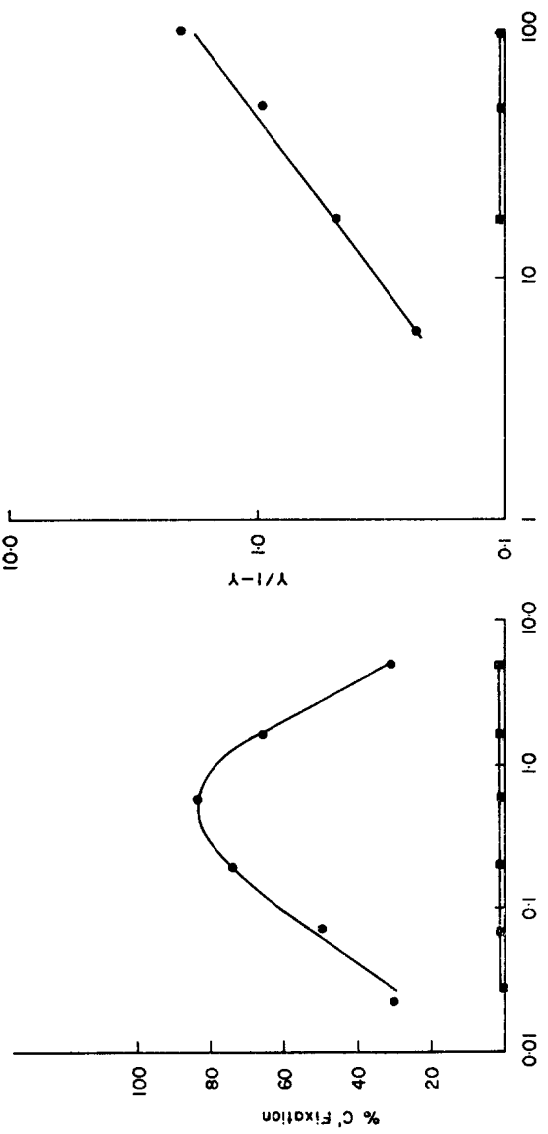


FIG. 7. (A) C′ fixation of RSA–glutaraldehyde–DOM (●) and RSA–glutaraldehyde–DMPE, RSA–glutaraldehyde–TMPE and RSA–glutaraldehyde–amphetamine (■) with anti-RSA–glutaraldehyde–DOM diluted 1/300. (B) C′ fixation inhibition of the reaction between 0·6 μg RSA–glutaraldehyde–DOM and anti-RSA–glutaraldehyde–DOM diluted 1/300 by DOM (●) and DMPE, TMPE and amphetamine (■).

DISCUSSION

The antibody synthesizing site is probably presented with the benzene ring and the methoxy groups, since in the antigenic conjugates the amino group of the phenylethylamine is part of the amide linkage to the macromolecule. The TMPE immune system was inhibited effectively with those phenylethylamine derivatives that possessed methoxy groups in the 3,4 and 5 positions of the benzene ring. Removal of any of these methoxy groups, as in DMPE, 3,5-dimethoxyphenylethylamine or 1-methoxyphenylethylamine, drastically decreased the ability of these compounds to function as effective inhibitors. The immune system was not sensitive to substitution of methyl groups for the hydrogens in the amino group of the side chain, since TMPE, *N*-methyl TMPE and *N*-dimethyl TMPE were equally effective inhibitors.

In the DMPE immune system, certain structural features are also required for a compound to be an effective inhibitor. The number and position of methoxy groups on the benzene ring are important. Veratric acid (3,4-dimethoxybenzoic acid) and DMPE are equally effective inhibitors. An additional methoxy group in the 5 position diminished the inhibitory effectiveness of a compound approximately 100-fold (compare TMPE derivatives with DMPE), while a loss of a methoxy group from the 4 position yields a noninhibitor. 3,5-Dimethoxyphenylethylamine is not inhibitory even at 500 times the concentration of DMPE. Replacement of the hydrogens of the primary amine with methyl groups has relatively little effect; mescaline, *N*-methylmescaline and *N*-dimethylmescaline, although poor inhibitors, are equally as effective in inhibiting the system. Replacement of the 3,4-methoxy groups with hydroxyl groups as in *l*-norepinephrine and Dopa prevents these compounds from exerting any inhibitory effect in the system.

Weil-Malherbe[15] has reviewed the claims that DMPE is present in the urine of schizophrenic patients. Its detection was based on the appearance of a "pink spot" on paper chromatograms which possess R_f values similar to DMPE and undergo characteristic color changes when sprayed first with ninhydrin reagent and then with Ehrlich's reagent. The criticisms against the pink spot assay for DMPE and the use of other more suitable assays have also been reviewed. Recent findings indicate that the pink spot is due to substances other than DMPE; it is not specific for schizophrenia and is of exogenous origin. In all three of the immune systems studied, compounds which can act as effective inhibitors must meet strict structural requirements and thus the immunological technique offers a potentially powerful assay tool to detect low concentrations of these drugs in biological fluids or tissues.

The cross tolerance which occurs between psychedelic drugs[16, 17] has suggested to some investigators that the compounds act on the same central receptor in the organism. In attempting to describe the steric factors that would predict psychotropic activity among tryptamine, phenylethhlamine and amphetamine derivatives, Snyder and Richelson[18] have observed that in molecular models the more active derivatives in each class could assume unique conformations which simulate in part the rings of lysergic acid (LSD). For example, with mescaline, intramolecular hydrogen bonding with pi electrons of the benzene C_2 or C_6 position and the hydrogen of the amine side chain resembles the indole ring of LSD. If antibodies can be made to LSD through the side chain so that the four ring structures are unaltered, it may be possible to test this hypothesis by observing the extent of inhibition caused by phenylethylamine derivatives bearing different substituents.

The specific antibodies may also prove to be potentially useful reagents in neutralizing the pharmacological effects that these substances possess *in vivo*. Ranadive and Sehon[12] were able to inhibit the cutaneous reactions in mice evoked by the intradermal injections of serotonin by using the specific antisera.

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